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Determination of sulfadoxine and pyrimethamine in microvolume human plasma using ultra high performance liquid chromatography-tandam mass spectrometry

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Abstract

To support the pharmacokinetic study of sulfadoxine (SD) and pyrimethamine (PM) in pregnant women and children, sensitive methods with small sample volume are desirable. Here we report a method to determine SD and PM with microvolume plasma samples: 5 μL plasma samples were cleaned up by protein precipitation with acetonitrile. The deuterated analytes were used as the internal standards. The samples after cleanup were injected onto an ACE Excel SuperC_{18} column (50 × 2.1 mm, 1.7 μm, Hichrom Limited) connected to a Waters I class UPLC coupled with a Sciex Triple Quad 6500$^+$ Mass Spectrometer and eluted with water and acetonitrile both containing 0.1% formic acid in a gradient mode at 0.8mL/min. Detection utilized ESI$^+$ as the ion source and MRM as the quantification mode. The precursor-to-product ion transitions m/z 311→245 for SD and 249→233 for PM were selected for quantification. The ion transitions for the corresponding internal standards were 315→249 for SD-d_4 and 254→235 for PM-d_3. The

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Author contributions

V.S. and L.H., Method development and validation; L.H. Method design; F.M., K.G., and L.H., clinical sample analysis; G.D., P.J. R., N.M. and F.A. Clinical study design; G.D., P.J.R., and F.A., funding acquisition; M.R. and J.L., Curation of database and clinical samples; V.S. and L.H., wrote the manuscript; All authors reviewed the manuscript.

CRediT authorship contribution statement

Vong Sok: Methodology, Validation, Writing – original draft. **Florence Marzan:** Investigation. **Michelle Roh:** Data curation, Writing – review & editing. **Kevin Guo:** Investigation, Writing – review & editing. **Jenny Legac:** Data curation, Resources, Writing – review & editing. **Norah Mwebaza:** Investigation, Resources, Supervision, Writing – review & editing. **Grant Dorsey:** Funding acquisition, Supervision, Writing – review & editing. **Philip Rosenthal:** Funding acquisition, Supervision, Writing – review & editing. **Francesca T. Aweeka:** Funding acquisition, Supervision, Writing – review & editing. **Liusheng Huang:**.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2024.124030.

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simplest linear regression weighted by 1/x was used for the calibration curves. The calibration ranges were 1–200 μg/mL SD and 2 – 1000ng/mL PM. The mean (± standard deviation) recoveries were 94.3±3.2% (SD) and 97.0±1.5% (PM). The validated method was applied to analysis of 1719 clinical samples, demonstrating the method is suitable for the pharmacokinetic study with samples collected up to day 28 post-dose.

Keywords

UHPLC-MS/MS; Sulfadoxine; Pyrimethamine; Plasma; Microvolume

1. Introduction

Malaria is a mosquito-borne disease caused by malaria parasites (Plasmodium strains). Each year the estimated cases of malaria are over 200 million leading to more than half a million deaths. In 2020, there were an estimated 241 million malaria cases and 627 000 malaria deaths worldwide, among which 77 % deaths were from children less than 5 years of age [1]. There were 34 % pregnant women in moderate/high transmission Africa region exposed to malaria infection, resulting in 819,000 children with low birthweight, a strong risk factor for neonatal and childhood for mortality. Intermittent preventive treatment in pregnancy (IPTp) reduces the rate of low birthweight and saves lives.

To support our pharmacokinetic (PK) studies of dihydroartemisinin-piperaquine and sulfadoxine-pyrimethamine for IPTp in Uganda, methods for quantitation of these drugs are needed. Previously our lab developed methods for quantitation of artemether/dihydroartemisinin [2] and piperaquine [3]. In this study, we aimed to develop a high through put microvolume method for determination of sulfadoxine (SD) and pyrimethamine (PM). The challenges for simultaneous quantification of SD and PM are (1) their disparate chemical properties and (2) high concentration ratio in clinical plasma samples. SD is a weak acid with the predicted pKa 6.16 ± 0.50, but PM is a weak base with the predicted pKa 7.18 ± 0.10 [4]. The concentration ratios of SD/PM in clinical samples could be > 100 [5–7]. Numerous methods using high performance liquid chromatography coupled with ultra-violet detectors (HPLC-UV) have been reported for quantification of SD/PM in plasma [8–12]. Due to low specificity of UV detection, complex sample preparation procedures such as solid-phase and liquid-phase extractions were used in these methods, the run times were typically over 10 min, and sample volumes were 500 μL or more. Several later methods were developed on HPLC coupled with Tandem Mass Spectrometry detectors (HPLC-MS/MS), which had higher sensitivity and specificity enabling simpler sample preparation and shorter run time [5,13]. But sample volumes were still ≥ 50 μL, and complex regression models were used for SD calibration curves. Although LC-MS/MS methods using dried blood spots (DBS) were reported for SD and PM to support adherence [14] and epidemiology studies [15], they were not fully accepted for drug quantification to support PK studies due to the uncertainty associated with DBS sampling methods. The gold standard methods for drug quantification are still LC-MS/MS methods based on plasma samples. We currently report a method using only 5 μL plasma samples, based on a highly sensitive ultra high performance liquid chromatography-tandem mass

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spectrometry (UHPLC-MS/MS) platform, and the calibration curves for both SD and PM were constructed with simple linear regression models with a 1/x weighting factor.

2. Experimental

2.1. Method design

In the ongoing clinical studies of sulfadoxine-pyrimethamine for IPTp in Uganda, plasma samples are collected up to day 28 post dose. Based on published PK studies [5–6,16–17], we estimate the day 28 drug concentrations are 10 μg/mL SD and 12 ng/mL PM with concentration ranges of 10–120 μg/mL SD and 12–750 ng/mL PM. Therefore, we set the calibration range of methods at 1–200 μg/mL SD and 2–1000 ng/mL PM.

2.2. Reagents and materials

Sulfadoxine and pyrimethamine reference standards used European Pharmacopoeia reference standards purchased from Sigma-Aldrich (St Louis, MO, USA). The internal standards (I.S.) sulfadoxine-d_4 and pyrimethamine-d_3 were purchased from Toronto Research Chemicals (Toronto, Canada) (Fig. 1). Acetonitrile (MeCN), methanol, water, ammonium formate, and formic acid were obtained from Fisher Scientific (Fair Lawn, NJ, USA). All solvents were Optima™ LC-MS grade and chemicals were of ACS certified grade. K_2EDTA human plasma and blood were purchased from Biological Specialty Co., (Colmar, PA, USA).

2.3. Instrumental and analytical conditions

The Waters Acquity UPLC system (I class) (Waters Co., Milford, MA, USA) coupled with Sciex TripleQuad 6500+ tandem mass spectrometry (MS/MS) managed with the software Analyst® 1.6.3. (Sciex Inc., Redwood City, CA, USA). Chromatographic separation was achieved on an ACE® Excel SuperC_{18} analytical column (50 × 2.1 mm, 1.7 μm) eluted with water (A) and acetonitrile (B) both containing 0.1 % formic acid at a flow rate of 0.8 mL/min in gradient mode. The gradient elution was programed as follows: 25 % solvent B (0–0.2 min), from 25 to 40 % B (0.2–1.0 min), 40–90 % B (1.0–1.1 min), 90 % B (1.1–1.5 min), from 90 to 25 % B (1.50–1.51 min), and 25 % B (1.51–1.6 min), injection volume was 3 μL. The divert valve was set to direct LC eluent to MS source at 0.3 min and to waste line at 1.2 min. The MS conditions are summarized in Table 1. Data was processed with Analyst 1.6.3. (Sciex Inc., Redwood City, CA, USA).

2.4. Preparation of standard and quality control samples

SD stock solutions were made in MeCN-water (1:1, v/v) and PM stocks were in MeCN-water (1:1, v/v) containing 0.5 %FA; working solutions are made in MeCN-water (1:1, v/v). The working solutions were spiked to blank K_2EDTA plasma to obtain calibration standards at the concentrations of 0.4/2, 1/5, 2/10, 5/25, 10/50, 20/100, 50/250, 100/500, and 200/1000 μg/mL SD / ng/mL PM. Calibration ranges are 1–200 μg/mL for SD (Std #2–9) and 2–1000 ng/mL for PM. QC samples consisted of 3/6, 16/80, and 160/8000 μg/mL SD / ng/mL PM. The I.S. working solution was prepared at 1000/10 ng/mL SD-d_4/PM-d_3 in MeCN-water (1:9, v/v) containing 0.1% formic acid. The stock solutions, standards, QC samples, and the I.S. solution were stored at −70 °C freezer between uses.

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2.5. Sample preparation

Five microliter (5 μL) plasma samples were pipetted into 20 μL I.S. (1000/10 ng/mL SD-d_4/PM-d_3) with tips submerged in the I.S. solution, mixed briefly, added 175 μL MeCN followed by vortex-mixing 5–10 sec and centrifuging at 20,000 rcf for 3 min. The supernatants were diluted with water by 5-fold and injected 3 μL into the UPLC-MS/MS system.

2.6. Method validation procedure

The method validation was conducted according to the guidelines of the NIH-sponsored Clinical Pharmacology Quality Assurance (CPQA) Program of the AIDS Clinical Trials Group (ACTG), and Food and Drug Administration (FDA) guidelines [19].

2.6.1. Calibration curve—The guidelines recommend the calibration curve should be fitted with the simplest regression model with an appropriate weighting factor [19]. Calibration curves were obtained by linear regression of the peak area ratio of analyte to internal standard (Y-axis) versus the nominal analyte concentrations (X-axis) with a weighting factor of 1/x. At least six (6) nonzero calibrators must be used to derive the standard curve. The back-calculated concentrations for calibrators should be within 15 % deviation of the nominal concentrations (20 % at LLOQ), and ≥ 75 % of calibrators must meet this criterium.

2.6.2. Lower limit of quantitation (LLOQ)—The LLOQ was established using five or more samples independent of standards to determine accuracy and precision. The accuracy should be within 20 % deviation from the nominal concentration and precision should be < 20 %. The signal to noise ratio at the LLOQ should be ≥ 5, and the signal intensity of the LLOQ should be ≥ 5-fold the response from the blank sample processed with the I.S.

2.6.3. Intra- and inter-day precision and accuracy—Intra-day precision and accuracy were determined by analysis of five or more replicates of each QC sample (n ≥ 5) at low (≤3-fold LLOQ), medium (in the middle of calibration range), and high (≥80 % upper limit of quantitation) concentration levels along with a set of calibrators in one batch. The same procedure was repeated on three (3) different days with new samples to determine inter-day precision and accuracy (total: n ≥ 15 per concentration level). Precision was reported as relative standard deviation (RSD) or coefficient of variation (CV%) and accuracy as percent deviation from the nominal concentration (dev %). The assay is considered acceptable if precision (CV%) is less than 15 % for intra and inter-day variation and accuracy (dev %) is within 15 % for intra- and inter-day comparison (dev % for LLOQ should be within ± 20 %).

2.6.4. Recovery and matrix effect—The recovery of analytes from plasma following sample preparation was assessed by comparing the peak area of analytes from plasma extracts (preextraction-spiked samples, set 3) to the peak area of the same concentration of analytes spiked into blank plasma extracts (postextraction-spiked samples, set 2). Matrix effects were evaluated by comparing the peak area of analytes from postextraction-spiked samples (set 2) to clean samples with analytes spiked in mobile phase solvents (set 1).

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Process efficiency was evaluated by comparing set 3 to set 1. The recovery, matrix effect, and process efficiency were calculated with the following formulae:

$$Recover(RE) = \frac{100 \times Peak\ \ area\ \ of\ \ preextraction - spiked\ \ sample(set3)}{Peak\ \ area\ \ of\ \ postextraction - spiked\ \ sample(set2)}$$

$$Matrix\ \ effect\ \ (ME) = \frac{100 \times Peak\ \ area\ \ of\ \ postextraction - spiked\ \ sample\ \ (set2)}{Peak\ \ area\ \ of\ \ clean\ \ sample\ \ (set1)}$$

$$Process\ \ efficiency(PE) = \frac{100 \times Peak\ \ area\ \ of\ \ preextraction - spiked\ \ sample(\text{set3})}{Peak\ \ area\ \ of\ \ clean\ \ sample(set1)}$$

Matrix effect was also evaluated with a slope analysis experiment according to CPQA guidelines [18], which was based on an approach proposed by Matuszewski et al. [20]: each of 6 different lots of blank plasma was spiked with analytes at the low, medium, and high concentrations. One set of the spiked samples at low, medium, and high concentration from each lot of plasma was processed and analyzed with the method. A slope was calculated from linear regression of the peak area ratio versus nominal concentration in each lot of plasma. The precision (CV%) of the slopes from the 6 lots of plasma should be ≤ 5 % according to the CPQA guidelines.

For selectivity, 6 different lots of blank plasma were also processed without the I.S. and injected into the LC-MS/MS system along with an LLOQ sample. The signals in the double blank samples should be ≤ 20 % of analyte signal at the LLOQ and ≤ 5 % of the I.S. signal.

Potential interference of possible concomitant drugs was also evaluated for the SD/PM assay by spiking the low and high QC samples of SD/PM with each of the potential concomitant drugs (n = 3). The spiked samples were analyzed as usual and compared with QC response without the concomitant drugs. The following drugs (concentrations) were tested: Artemether /dihydroartemisinin (500 ng/mL), piperaquine (1 ug/mL), lumefantrine (20 ug/mL), desbutyllumefantrine (1 ug/mL), efavirenz (5 μg/mL), tenofovir/emtricitabine (1 μg/mL), lopinavir (5 μg/mL), ritonavir (1 μg/mL), nevirapine (5 μg/mL), and dolutegravir (1 μg/mL).

2.6.5. Stability—The stability of analytes in human plasma was evaluated at these conditions: Three or more freeze–thaw cycles, storage at −70 °C and room temperature (22 °C). Each condition was tested with QC samples at low and high concentration levels in triplicates. Fresh samples were used as reference. Stability of processed samples was evaluated by reinjecting the processed samples after staying in the autosampler for four days.

2.7. Application to clinical studies

The method has been applied to two clinical studies. One study was to compare dihydroartemisinin-piperaquine and SD-PM as IPTp in pregnant women (“DPSP” study) in Busia, Uganda. Initially, 179 samples were collected ~ 2hr post-dose to evaluate safety. Then, 942 samples were collected for intensive PK study at 0, 0.5, 1, 2, 3, 4, 6, 8 hr, and 1, 2, 3, 7, 9, 16, 23 days post-dose from 60 pregnant women after 28 weeks of gestation. Lastly, 391 trough samples were collected around day 28 post-dose prior to next dose at 24 and 32 weeks of gestation. Another study was a seasonal malaria control study with SD-PM

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and artesunate-amodiaquine in children aged 6–59 months in Burkina Faso ("DRUMARS" study). Plasma samples (n = 207) were collected at the time of malaria diagnosis in children (n = 104) and the control children (n = 103) from the same health facility with a nonmalarial diagnosis on the same days. Both studies were approved by the institutional review boards of Makerere University in Uganda, Institut des Sciences et Techniques in Burkina Faso, and University of California San Francisco in USA, and informed consent forms were signed by participants or guardians.

3. Results

3.1. LC-MS/MS optimization

The UHPLC-MS/MS system was optimized in both APCI+ and ESI+ modes. ESI^+ was chosen for its higher sensitivity. The UPLC system enables application of sub-2 μm LC columns, which significantly increase column efficiency and resolution, improve assay sensitivity, and reduce analytical time [21]. To optimize LC separation, several columns for LC separation were tested using water and MeCN as mobile phase solvents both with 0.05 %TFA or 0.1–0.5 % FA and with/without NH_4FA. SD and PM were only partially separated on C_8 (50 × 2.1 mm, 1.8 um, Agilent Tech); PFP (30 × 2.1 mm, 1.8 um, Waters) gave tailing peaks for both SD and PM; PFP (50 × 2.0 mm, 3um, Agilent Tech) and T_3 (75 × 2.1 mm, 1.8um, Waters) yielded good peak for SD but tailing peak for PM. ACE Super C_{18} (50 × 2.1 mm, 1.7 um, Hichrom Ltd) yielded good separation and sharp peaks for both analytes, thus it was used in this assay. To avoid cross talks from analyte, the less abundant ion from the ^{37}Cl isotope (i.e. the most abundant ion plus 2) was selected for the deuterated I.S. PM-d_3. Concentrations of PM in clinical samples are often < 1000 ng/mL but SD > 1000 ng/mL [22], which presented a challenge to simultaneous quantification of PM and SD. To avoid signal saturation due to high SD concentration, we increased the decluster potential from 60v to 200v to desensitize the SD signal, however, the linear range of SD calibration curve was too narrow. Then we chose a less sensitive SD product ion *m*/*z* 245, which is about 10 % of signal from the most abundant product ion *m*/*z* 156. By doing so, the simplest linear regression with a 1/x weighting factor was able to be used for both SD and PM calibration curves.

Representative mass spectra of SD, PM, and I.S. are shown in Fig. 1.

3.2. Sample preparation

A simple protein precipitation was used. Owing to the highly sensitive UHPLC-MS/MS system, we only used 5 μL plasma samples to process and injected 3 μL of the processed samples which were 200-fold diluted from plasma samples.

3.3. Method validation

3.3.1. Calibration curve linearity—The calibration ranges were 1–200 μg/mL SD and 2–1000 ng/mL PM. With simple linear regression weighted by 1/x, the calibration curve had a mean coefficient of determination r^2 of 0.9993 ± 0.0001 for SD and 0.9992 ± 0.0002 for PM (Table 2). Of note, SD concentration was 200-fold higher than PM in each calibrator, linear regression was only feasible with the less abundant SD product ion *m*/*z* 245. The

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LLOQ was 1 μg/mL SD (S/N = 92) and 2 ng/mL PM (S/N = 9). No carry over was observed in the blank plasma injected after the upper limit of quantitation. Representative MRM ion chromatograms of blank plasma and LLOQ sample are shown in Fig. 2.

3.3.2. Precision and accuracy—The intra-day precisions (n = 6) over 3 days were ranged from 1.9 to 5.4 % for SD and 1.8–8.4 % for PM at the three concentration levels, and inter-day precisions were ranged from 4.5 to 7.2 % for SD and 5.4–8.2 % for PM, all of them within 15 %. The intra- and inter-day accuracies were all within ± 15 %. The intra-day and inter-day precisions and accuracies at LLOQ levels were within 20 % and ± 20 %, respectively, all within the acceptance limit (Table 3).

3.3.3. Dilution integrity—Diluting plasma-spiked SD/PM 400/4 μg/mL with plasma by 10 folds and analyzed in five-replicates against freshly prepared calibrators and QCs yielded a 5.5 % and 3.5 % concentration deviation for SD and PM, respectively, when compared to the derived nominal concentrations of 40/0.4 μg/mL, indicating up-to-10-fold dilution integrity of SD and PM.

3.3.4. Recovery, matrix effect and selectivity—The results for recovery and matrix effect were shown in Table 4. The recoveries were very high, ranging from 91.7 to 97.8 % for SD and 95.3 – 98.2 % for PM. The IS-normalized matrix effects were 89.1, 93.6, and 102 % for SD and 84.0, 90.6, and 102 % for PM at low, medium, and high QC concentrations, respectively (Table 4).

To further evaluate matrix effect, we spiked SD and PM in 6 different lots of K_2EDTA human plasma and two lots of K_3EDTA human plasma at low, medium and high concentrations according to the CPQA guidelines (ref 2017). The samples were processed along with calibrators. The CV% of the slopes from linear regression of low, med, and high concentrations in the 6 lots of plasma were 2.6 % for SD and 1.1 % for PM, both < 5 %, suggesting no significant matrix effect or quantitative bias resulting from the matrix in the MS ionization source. However, QC-low 6 ng/mL PM from plasma lot# 1 gave abnormally high signal: the peak area ratio was 0.655 while other 7 lots of plasma at the same PM concentrations had values ranged from 0.272 to 0.324. Dixon test suggested it is an outlier, but slope analysis still yielded satisfactory result (<5% CV) (Supplemental material Table S1). This data suggests matrix effect evaluation with the approach proposed by Matuszewski et al is not reliable [20], although it has been used widely and adopted by CPQA guidelines [18].

To evaluate selectivity, 8 different lots of blank human plasma were processed and analyzed along with a LLOQ sample: the signals at the retention times of SD and PM were all less than 20 % of LLOQs, and the signals at the retention times of ISs were negligible (<5% of IS signal) (Data not shown).

3.3.5. Stability—Results for stability in human plasma (samples) and solution (stocks) were shown in Table 5. The stock solutions of SD in 50 % MeCN and PM in 50 % MeCN 0.5 %FA were stable in freezer (−70 °C) for at least 352 days and at room temperature (22 ± 3 °C) for at least 44hr. We also observed that 8 mg/mL SD in 50 % MeCN 1 % HCl were

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stable at room temperature for at least 2 months (data not shown). When the plasma samples stood on bench (22 ± 3 °C) for up to 6 days, there was no significant degradation observed for both SD and PM. They were also stable in freezer (−70 °C) for at least 960 days and 4 freeze–thaw cycles. After processing, SD and PM were stable in autosampler for 102 hr. In summary, SD and PM are very stable drugs in both solution and plasma.

To test stability of SD and PM in whole blood, we spiked the drugs in blank K_2EDTA blood at 3/6 and 160/800 μg/mL SD / ng/mL PM, centrifuge aliquots of the bloods to yield plasma at 0.5, 2,4, and 30 hr. The resulting plasma and red blood cells were analyzed in triplicate along with a set of calibrators and 2 sets of QCs. Compared to the plasma samples yielded at 0.5 hr, the change of concentrations for both SD and PM were < 5 %, but SD concentrations were higher than the nominal values (+70 % at 3 μg/mL and + 40 % at 160 μg/mL), suggesting SD concentrated in plasma. The erythrocyte-plasma partition ratio is concentration dependent (0.14 at 5 μg/mL and 0.38 at 224 μg/mL SD) (Supplemental Table S2).

3.3.6. Impact of hemolysis—To evaluate impact of hemolysis, whole blood underwent 2-freeze–thaw cycles to lyse the erythrocytes. The lysed blood was spiked into plasma at a ratio of 5:95 to yield 5 % hemolyzed plasma, which was spiked with SD/PM at low and high concentrations and analyzed in triplicate along with a set of calibrators and 3 sets of QCs as the reference. The difference between the hemolyzed plasma and reference plasma at low and high drug concentrations were −2.2 % and −6.1 % for SD, −6.9 % and −2.0 % for PM. The results suggest lysed erythrocytes did not impact the quantitation of SD and PM. However, in view of uneven distribution of SD in blood, hemolysis should be avoided or minimized.

3.3.7. Impact of concomitant drugs—We tested selectivity of the method for SD and PM over other potential concomitant antimalarial and HIV drugs. The peak area ratios of the concomitant drug-spiked samples were compared with that of non-spiked control sample at low and high concentrations. The data shows all tested drugs did not affect this assay: artemether, dihydroartemisinin, piperaquine, lumefantrine, desbutyllumefantrine, efavirenz, lopinavir, ritonavir, nevirapine, dolutegravir, tenofovir, and emtricitabine. Therefore, those drugs, if used by patients, would not affect quantification of SD and PM. (Supplemental material Table S3).

3.4. Application

The method was used to analyzed 1719 clinical plasma samples from two studies. Among the 207 samples from DRUMARS study conducted at Burkina Faso, 81 samples for SD and 46 samples for PM were quantifiable The detailed clinical results have been reported elsewhere [23].

Among the 1512 samples from the DPSP study, the initial 179 samples collected at 2 hr post-dose were all within the calibration range, with the median (range) concentrations at 116 (42.9, 180) μg/mL SD and 306 (94.4, 564) ng/mL PM., Among the 942 intensive PK samples collected from 0 to 23 days post-dose, 12 samples for SD and 6 samples for PM were below LLOQ. The maximum concentrations were 162 μg/mL SD and 768

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ng/mL PM, both within the calibration ranges. Among the 391 trough samples collected around day 28 post dose, 28 SD concentrations and 11 PM concentrations were < LLOQ. The median (range) day 28 concentrations were 5.26 (BLLOQ, 34.9) μg/mL SD and 11.0 (BLLOQ, 105) ng/mL PM, with BLLOQ representing below LLOQ. A representative plasma concentration–time curve from a pregnant woman is shown in Fig 3. The full clinical PK data for DPSP study will be reported elsewhere.

The incurred sample reanalysis confirms the method is reliable (Supplemental Table S4). The differences between the two analyses meet the criteria of < 20 % for at least two-third of samples: 137 of 142 reanalyzed samples (96 %) for SD and 129of 138 reanalyzed samples (93 %) with quantitiable PM concentrations had less than 20 % difference from the initial analysis.

4. Discussion and conclusion

There are several findings and improvements in this report. First, most methods utilized conventional 3–5 μm columns including C_{18} [8, 24], phenyl [13,22], or T_3 [15]. We used the Excel SuperC_{18} sub-2 μm UHPLC column for better separation efficacy and resolution, enabling the analytical time per sample shortened to under 2 min. Second, the clinical relevant concentrations of SD and PM are spanned over a large range with SD at the high end (1–200 μg/mL) and PM at the lower end (2–1000 ng/mL), making it a challenge to simultaneous quantification of the two drugs. Previously published methods used quadratic fitting for SD [13,15], which often caused overestimate of medium and high concentrations if insufficient calibrators included, or linear fitting with $1/x^2$ weighting factor [22], which lead to overestimate of higher concentration. By choosing the less abundant product ion, we were able to fit the calibration curve with a simple linear regression and 1/x weighting factor, making the methods more robust. Removal of one to two calibrators won't cause significant impact on the assay performance. Third, we found background signal formed an interference peak at the retention time of PM in the cases of high baselines, initially we thought it was a carryover residual PM peak. But when we increased initial mobile phase solvent B from 20 to 25 %, the interference peak was resolved. Fourth, addition of acid to the I.S. solution is critical for consistent signal of PM-d_3. When samples were processed with I.S. in water or water-MeCN (9:1, v/v), we observed consistent SD-d_4 signal but significantly varied PM-d_3 signal, which did not impact assay performance as confirmed by QC samples and incurred sample re-analysis (Supplemental Table S4). When samples were processed with I.S. in water-MeCN (9:1, v/v) containing 0.1% formic acid, consistent PM-d_3 signals were observed.

Application of the method to analysis of clinical samples collected in pregnant women receiving IPTp was successful. Among the 391 trough day 28 samples, 28 (7 %) for SD and 11 (3 %) for PM were under LLOQ. The total BLLOQ samples among the 1512 samples from pregnant women were 40 (2.6 %) for SD and 17 (1.1 %) for PM. The results confirm the validated method is suitable to support the intended PK study with samples collected up to 28 days post-dose. Application of the method to clinical samples collected in children receiving seasonal malaria chemoprevention yielded over 50 % drug levels below LLOQ. As drug administration was based on parent/guardian recall, some children might not take the

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medicine or vomited during drug administration. Still, detectable peaks were presented in 9 % samples under LLOQ for SD and 12 % samples under LLOQ for PM. Thus, the study in children could benefit from a more sensitive method.

In summary, a high-throughput UHPLC-MS/MS method was developed for quantification of SD and PM simultaneously. The method utilized only 5 μL plasma samples and the run time is only 1.6 min per sample. The method has been successfully used to analyze over 1500 clinical samples. The method can be combined with capillary microsampling method for blood collection to facilitate PK studies in rural areas and pediatric population.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability

Data will be made available on request.

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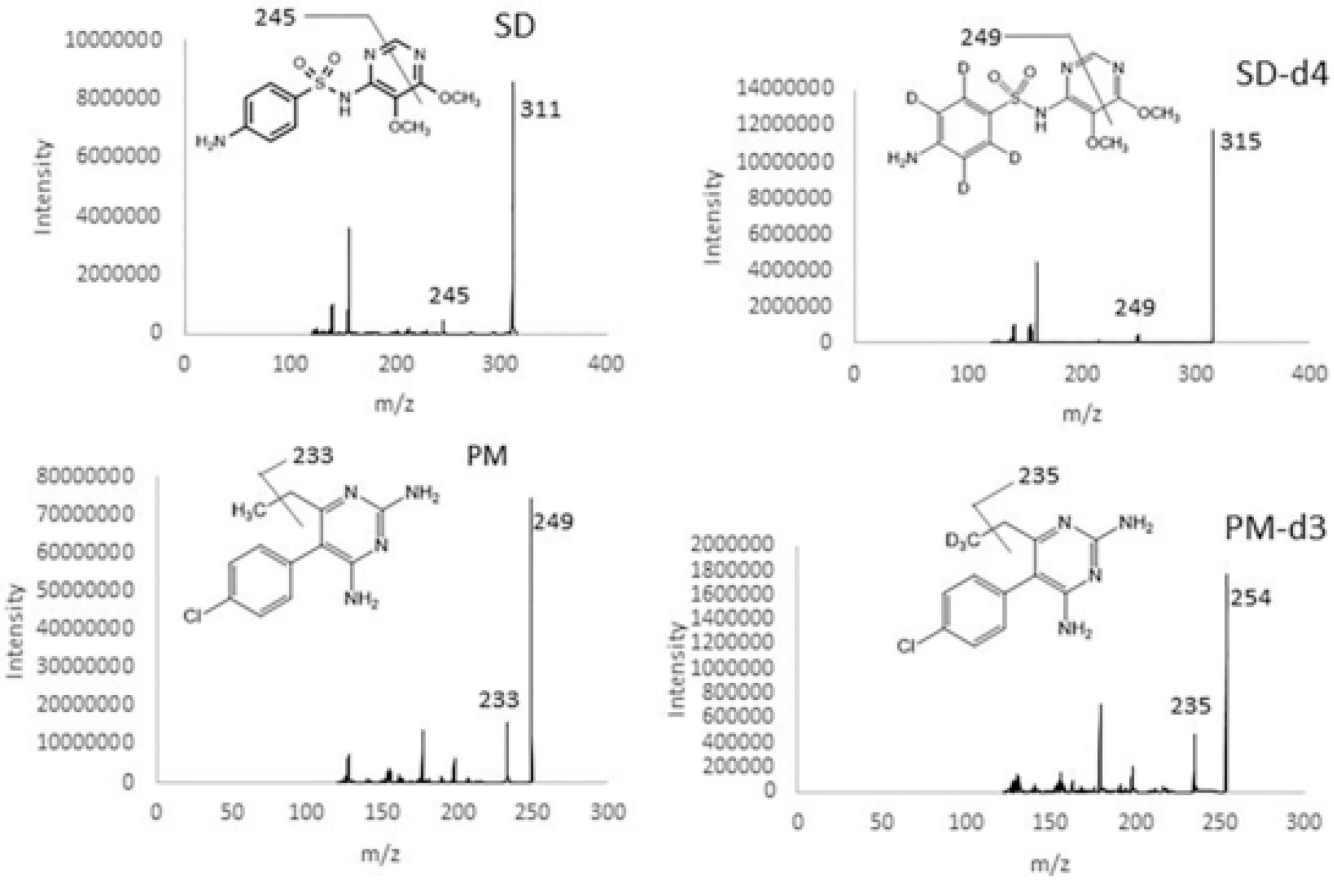

Fig. 1.

Representative mass spectra of SD, PM, and the internal standard SD-d_4 and PM-d_3.

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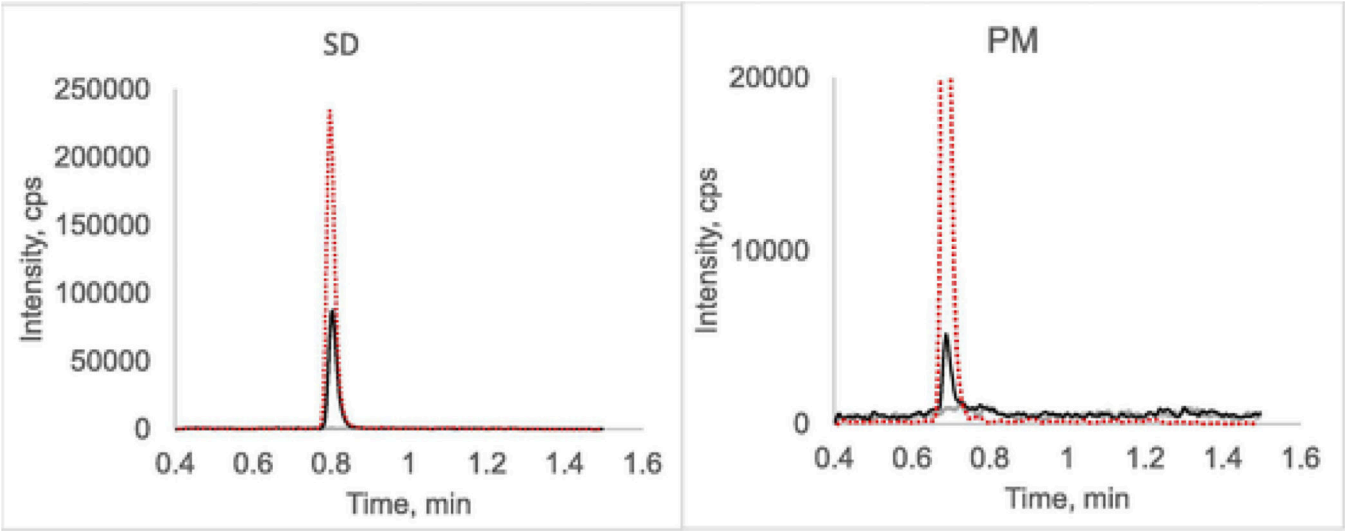

Fig. 2.

Representative MRM ion chromatograms of blank plasma (grey) and an LLOQ sample (black). The red dash line represents the I.S. The left panel is for SD, right panel is for PM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

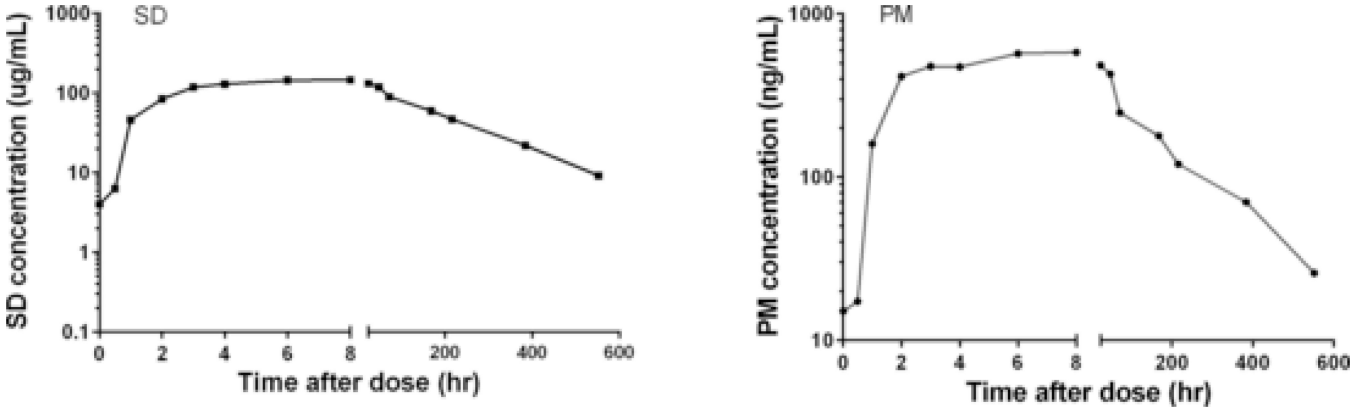

Fig. 3.

A representative concentration-time profile of SD and PM in plasma from a pregnant woman.

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Table 1

Optimized MS/MS parameters.

Source parameters	**TEM,**	**IS,**	**CAD,**	**CUR,**	**Gas1,**	**Gas2,**
	°C	**v**	**psi**	**psi**	**psi**	**psi**
	400	**2000**	**9**	**25**	**30**	**50**
Compound parameters	DP, v	EP, v	CE, v	CXP, v	Dwell time, ms	
311/245 (SD)	60	10	25	10	50	
315/249 (SD-d4)	60	10	25	10	50	
249/233 (PM)	60	10	41	12	50	
254/235 (PM-d3)	60	10	41	12	50	

TEM, source temperature; IS, ionspray voltage; CUR, curtain gas, Gas1, nebulizer gas; gas2, auxiliary gas; CAD, collision-activated dissociation; DP, declustering potential; EP, entrance potential; CE, collision energy; CXP, collision cell exit potential.

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Table 2

Inter-day average back-calculated standard concentrations (n = 6).

	SD								**R^2**
Nominal, μg/mL		**1**	**2**	**5**	**10**	**20**	**50**	**100**	**200**	
Mean conc.		0.883	1.95	5.12	10.7	20.7	50.8	101	197	0.9993
CV, %		5.3	1.8	3.1	4.5	0.8	3.1	0.8	0.7	
Dev, %		−11.7	−2.6	2.4	7.3	3.7	1.7	0.6	−1.4	
n		6	6	6	6	6	6	6	6	
	PM									**R^2**
Nominal, ng/mL	**2**	**5**	**10**	**25**	**50**	**100**	**250**	**500**	**1000**	
Mean conc.	1.91	4.99	10.3	25.3	50.4	101	246	506	997	0.9992
CV, %	7.6	6.2	6.6	3.3	5.8	1.2	1.9	3.5	2.7	
Dev, %	−4.4	−0.3	2.5	1.0	0.9	1.3	−1.5	1.1	−0.4	
n	5	6	4	6	6	6	6	6	6	

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Table 3

Intra-day and inter-day precision (CV, %) and accuracy (dev, %) for analysis of sulfadoxine and pyrimethamine in human plasma.

		Intra-day				Inter-day			
SD	Conc., μg/mL	1	3	16	160	1	3	16	160
	CV, %	4.2–5.5	2.8–5.4	1.9–3.9	2.5–2.8	7.4	7.2	4.5	5.8
	Dev, %	−19.9–(−8.2)	−7.6–7.1	5.8–13.6	−6.5–5.8	−13.1	−0.4	8.6	−0.9
	n	6	6	6	6	18	18	18	18
PM	Conc, ng/mL	2	6	80	8000	2	6	80	8000
	CV, %	4.6–19.8	7.5–8.4	1.8–6.9	3.0–4.2	13.1	8.2	7.4	5.4
	Dev, %	0.25–4.4	0.78–8.0	0.13–14.4	−2.1–7.7	2.69	5.0	8.21	3.28
	n	6	6	6	6	18	18	18	18

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Table 4

Matrix effect (ME) and recovery (RE).

	conc.	Peak area (x10^5), n = 3			ME	IS- normalized ME	RE
		unextracted	postextraction	preextraction			
		(in 10 %ACN 0.5 %FA)	spiked	spiked			
SD	Low	3.24 ± 0.05	3.21 ± 0.13	3.14 ± 0.24	99.0	89.1	97.8
	Med	17.5 ± 0.7	16.8 ± 0.7	15.7 ± 0.4	96.0	93.6	93.4
	High	151 ± 5	156 ± 6	143 ± 4	103	102	91.7
PM	Low	0.304 ± 0.006	0.246 ± 0.028	0.239 ± 0.043	80.7	84.0	97.4
	Med	3.86 ± 0.25	3.12 ± 0.55	2.98 ± 0.30	80.9	90.6	95.3
	High	38.5 ± 2.0	29.5 ± 3.9	29.0 ± 4.2	92.2	102	98.2

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Table 5

Stability of sulfadoxine and pyrimethamine.

		conc	%remained	CV%
4 fr-th	SD	low	95.6	0.4
		high	89.5	0.8
	PM	low	99.1	1.5
		high	96.2	1.7
22 ± 3 °C, 144hr	SD	low	95.8	6.0
		high	99.0	2.9
	PM	low	96.7	5.3
		high	98.0	3.3
22 ± 3 °C, 102 hr	SD	low	93.0	1.9
(processed)		high	94.0	2.0
	PM	low	105	14
		high	102	1.7
−70 °C, 960 d	SD	low	98.4	2.1
		high	95.5	4.0
	PM	low	90.9	4.1
		high	101	1.1
−70 °C, 352 d	SD		104	1.5
Stock	PM		105	4.7
22 ± 3 °C,44 h	SD		101	0.1
Stock	PM		102	0.16